

ESTIMATION OF ENZYME ACTIVITIES FOR THE DETOXIFICATION OF MALACHITE GREEN BY *Chlorella pyrenoidosa*

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ABSTRACT

The textile industries pose a serious threat to human and aquatic lives by the usage of azodyes as they offer an extensive range of colours, better colour fastness and four times the intensity of the azo free dyes. This challenge can be solved by decolorizing the dye by employing *Chlorella pyrenoidosa*. Apart from decolorisation, textile industries discharge the azo dyes directly in water which tends to cause environmental issue. The enzymes such as azoreductases, ascorbic acid peroxidase (APX) and superoxide dismutase (SOD) when released by *Chlorella pyrenoidosa* reacting with dye reduce the concentration of azodyes into simpler compounds. The enzyme activities were performed with the algal slurry after large scale expansion of the *Chlorella pyrenoidosa*. The enzyme extract was prepared and the activities were recorded. In the APX, SOD activities, the *Chlorella pyrenoidosa* was able to release the enzymes but as the dye concentration was higher, the enzyme activity was lower. In the azoreductase activity, increase in the concentration of the dye increased the enzyme activity. At 50 ppm, the enzyme activity was found as 1.616 millimolarunits/ ml enzyme. Efficiency of enzyme producing capacity for the reduction of azodyes toxicity was evaluated by release of azoreductases by *Chlorella pyrenoidosa*.

KEYWORDS: *Chlorella pyrenoidosa*, Azo dyes, Azoreductases, Ascorbic Acid Peroxidase, Superoxide Dismutase

INTRODUCTION

Chlorella pyrenoidosa

Chlorella pyrenoidosa is green algae that grow in fresh water. *Chlorella pyrenoidosa* has the largest amount of chlorophyll of any known plant. It contains all the essential amino acids along with provitamin A, various B vitamins, vitamin C, vitamin K, choline, inositol and lipoic acid (Mursaleen Usmani, 2014). *Chlorella pyrenoidosa* also contains the minerals calcium, copper, iodine, iron, magnesium and phosphorus, and a substance called chlorella growth factor (Jahir Alam Khan et al., 2011).

AZODYES

Azo dyes are known to be a very important and widely used class of toxic and carcinogenic compounds. Azo dyes account for approximately 60-70% of all dyes used in textile manufacture. **Azo dyes** are synthetic colours that contain an azo group, -N=N-, as part of the structure (Patrícia A. Ramalho et al., 2005).

TOXICITY OF AZO DYES

Azo dyes that are toxic only after reduction and cleavage of the azolinkage to give aromatic amines, mostly via

intestinal anaerobic bacteria. The aromatic amines are meta-bolically oxidized to reactive electrophilic species that covalently bind DNA (K.V. Ajayan and M. Selvaraju, 2012). Azo dyes with structures containing free aromatic amine groups that can be meta-bolically oxidized without azoreduction. Azo dyes that may be activated via direct oxidation of the azo linkage to highly reactive electrophilic diazonium salts (Gupta Shilpi et al., 2015).

MALACHITE GREEN

Malachite green is an organic compound that is used as a dyestuff. Malachite green, a triarylmethane dye (C₂₃H₂₆N₂O, CI 42,000) is a dark green and crystalline solid prepared by condensing one part of benzaldehyde with two parts of diethylaniline in the presence of concentrated sulphuric acid or zinc chloride (Hanan Hafez Omar., 2008).

EFFECT OF MALACHITE GREEN

Malachite green is environmentally persistent and acutely toxic to a wide range of aquatic and terrestrial animals. It causes serious public health hazards and also poses potential environmental problems (Robinson, T et al., 2001). This dye is highly toxic to freshwater. Malachite green also acts as a respiratory enzyme poison and causes respiratory distress in rainbow trout (Hanan Hafez Omar., 2008).

ENZYME ACTIVITY AND CHLORELLA PYRENOIDOSA

Physical and chemical treatment methods are common for the degradation of dye which includes precipitation, coagulation, adsorption, flocculation, flotation, electrochemical destruction, and mineralization and decolorization process (Gogate and Pandit, 2004) have some disadvantages such as cost, time, and release of residues. All these techniques are minimizing the toxicity level not to neutralize the toxicity. Algae reduce the azo dyes by secreting enzymes such as laccase, azo reductase, peroxidase, and hydrogenase (Ratna, B.S. Padhi., 2012). The reduced forms of azo dyes are further mineralized into simpler compounds and are utilized as their energy source (M. Sudha et al., 2014). Azo reductases are membrane bound enzymes that catalyze the reaction only in the presence of reducing equivalents like FADH and NADH. So the reduction process is taken place in bacterial cells with intact cell membranes (Robinson *et al.*, 2001).

MATERIALS AND METHODS

GROWTH OF *Chlorella pyrenoidosa*

The *Chlorella pyrenoidosa* was initially grown in the specific medium, Fog's medium and further used for the expansion.

EXPANSION OF *Chlorella pyrenoidosa*

The *Chlorella pyrenoidosa* which were cultivated was grown and then expanded in the 25 litres Bubble tops in order to get the huge production of algae. The medium used for the growth of *Chlorella pyrenoidosa* was Fog's medium. The *Chlorella pyrenoidosa* were grown in the Bubble tops and the algal biomass was collected.

FLOCCULATION

Flocculation is a reliable method for algae separation. Aluminium Chloride was used as a flocculant and after gravity clarification removed up to 85% of the suspended biomass from the high rate oxidation pond (Shivaji Srivastava et al., 2004). Various algae species could be separated by this reliable method to give algae slurry of 1.5%.

PREPARATION OF ENZYME EXTRACT

The flocculated algal Biomass were homogenized at 4°C in an prechilled mortar and pestle with liquid nitrogen in 100 mm potassium phosphate buffer (pH 7.8), 1mm EDTA, 1% triton x 100, 15% glycerol, with 50 mg of PVP per gram of biomass. Crude homogenates was centrifuged at 15,000 rpm 15 minutes at 4°C. Supernatant was frozen at -20° C (Bafana A et al., 2008).

SUPEROXIDE DISMUTASE

Superoxide dismutase was generated by xanthine oxidase and detected by nitro blue tetrazolium (NBT) reduction method. Reagents in this study are prepared with 50mM potassium phosphate potassium hydroxide buffer (pH 7.8). The reaction buffer should contain 50 µl of 0.6 mM NBT, 20 µl of 15 mM Na₂EDTA(pH 7.4), 30 µl of xanthine oxidase solution, 150 µl of enzyme sample and 1.5 µl of potassium phosphate potassium hydroxide buffer is used as control. Reaction was initiated by the addition of xanthine oxidase at 25° C. The absorbance was recorded at 405 nm in UV visible spectrophotometer. One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction under the assay conditions (Beauchamp, C. and I. Fridovich, 1971). Results were expressed as % of inhibition relative to control, given by

$$\frac{\text{Rate of control} - \text{Rate of sample reaction}}{\text{Rate of control}} \times 100\%$$

ASCORBIC ACID PEROXIDASE (APX)

APX extraction was performed in 1.5 ml of suspension solution including 50 mMTris-HCl (pH 7.2), 2 % PVP, 1 mM Na₂EDTA, and 2 mMascorbate. Assay solution contained 50 mM potassium phosphate buffer (pH 6.6), 2.5 mMascorbate, 10 mM, H₂O₂ and enzyme containing 100 µgprotein in a final volume of 1 ml. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate at 290 nm($\epsilon = 2.8 \text{ mM cm}^{-1}$ at 290 nm). The activity was expressed as U mg proteins⁻¹ (Nakano, Y. and K. Asada, 1981). It was calculated by using the formula,

$$\text{Volume activity} = \frac{\text{Total Volume (3 ml)} \times \text{Absorbance (290 nm)}}{\text{Ext. Coefficient (0.0028)} \times \text{Dilution Factor (1)} \times \text{Sample (1)} \times \text{Incubation time (1)}}$$

$$\text{Specific Activity} = \frac{\text{Volume activity}}{\text{Protein Concentration}}$$

AZOREDUCTASE ACTIVITY

Assay for azoreductase activity was performed using the following materials: 1. Potassium phosphate buffer 50mM (Reagent A). 2. Azo dye solution 0.1%, (Reagent B) 3. Enzyme solution (Reagent C) 4.Reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH) 2 mM (Reagent D). The assay procedure is based on the principle that with the addition of NADH to the reaction mixture containing substrate, buffer and enzyme solution, the substrate azo

dyes azo bond is degraded and there is a decrease in the absorbance of the dye after an initial lag phase. Unit Definition: One unit will reduce 1.0 μ mole of azo dye per minute in the presence of NADH using Millimolar extinction coefficient of azo dye at pH 7.0 and 30°C temperature

The procedure adopted was based on the one done by Zimmermann et al. (1982).

- **Pipetted (in Milliliters) the following Reagents into Test Tubes**

	Test	Blank
Reagent A (Buffer)	2.80	2.80
Reagent B (Azo dye solution)	0.05	0.05
Reagent C (Enzyme solution)	0.1	0.1(Distilled water)

P+++Mixed by inversion and equilibrate at room temperature. Then add:

Reagent D (NADH)	0.05	0.05
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- Immediately mixed by inversion and recorded the decrease in absorbance at 532 nm for approximately 5 min. obtained the ΔA 532 nm/ minute for both the Test and Blank.

CALCULATIONS

$$\text{Units/ml enzyme} = \frac{A_{532\text{nm}} / \text{min Test} - \Delta A_{532\text{nm}} / \text{min Blank} (3) (df)}{(\Delta B \times (0.1))}$$

3 = Total volume (in milliliters) of assay; df = Dilution factor (1); B= Millimolar extinction coefficient of azo dye Malachite green (1.5×10^5); 0.1 = Volume (in milliliter) of enzyme used

RESULTS AND DISCUSSIONS

GROWTH OF *Chlorella pyrenoidosa*

The growth of *Chlorellapyrenoidosa* was recorded at 660 nm using UV-Vis Spectrophotometer for a period of 10 days.

Table 1: Growth of *Chlorella pyrenoidosa*

Days	OD (660 nm)
1	0.0525
2	0.0944
3	0.1287
5	0.4602
6	0.5974
7	0.7679
8	0.8648
9	0.966
10	1.026

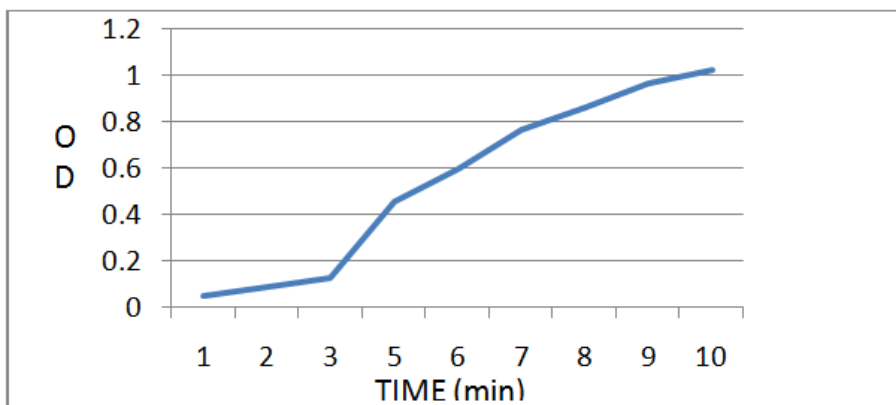


Figure 1: Graph Showing the Growth of *Chlorella pyrenoidosa*

COLLECTION OF BIOMASS

The biomass was collected at the end of flocculation by large expansion of *Chlorella pyrenoidosa* obtained from the bubble tops by allowing the algae to settle at the bottom.



Figure 2: Algal slurry of *Chlorella pyrenoidosa*

ENZYME ACTIVITY BY *Chlorella pyrenoidosa*

ASCORBIC ACID PEROXIDASE (APX) ACTIVITY

Table 2: Estimation of Ascorbic Acid Peroxidase in Dye by *Chlorella pyrenoidosa*

Dye Concentration with Chlorella	Enzyme Activity (U/mg Protein)
Control (Chlorella)	6.9
10 ppm	7.4
20ppm	13.2
30 ppm	13.8
40 ppm	12.9
50 ppm	11.3

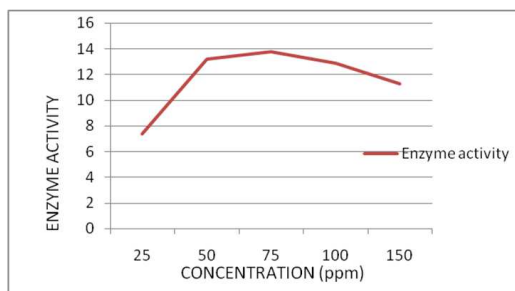
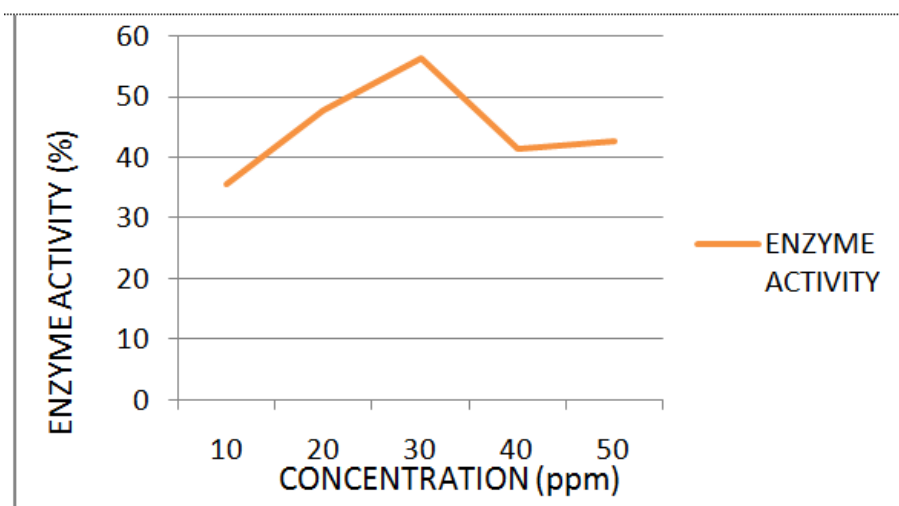


Figure 3: Graph Estimation of Ascorbic Acid Peroxidase by *Chlorella pyrenoidosa*

SUPEROXIDE DISMUTASE (SOD)**Table 3: Estimation of Superoxide Dismutase in Dye by *Chlorella pyrenoidosa***

Dye Concentration with Chlorella	Enzyme Activity (%)
10 ppm	35.61
20 ppm	47.82
30 ppm	56.30
40 ppm	41.39
50 ppm	42.69

**Figure 4: Graph Estimation of Superoxide Dismutase by *Chlorella pyrenoidosa*****AZOREDUCTASE ACTIVITY IN DYE BY *Chlorella pyrenoidosa*****Table 4: Estimation of Azoreductase in Dye by *Chlorella pyrenoidosa***

TIME (s)	ABSORBANCE					
	BLANK	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm
10	0.0254	0.0220	0.0259	0.0473	0.0747	0.0969
20	0.0255	0.0220	0.0257	0.0481	0.0753	0.0988
30	0.0248	0.0217	0.0252	0.0481	0.0768	0.0954
40	0.0254	0.0234	0.0254	0.0485	0.0747	0.0941
50	0.0255	0.0218	0.0268	0.0476	0.0756	0.0943
60	0.0276	0.0241	0.0257	0.0473	0.0747	0.0943
70	0.0262	0.0241	0.0252	0.0480	0.0747	0.0923
80	0.0262	0.0241	0.0248	0.0490	0.0757	0.0926
90	0.0256	0.0218	0.0252	0.0484	0.0763	0.0912
100	0.0255	0.0231	0.0260	0.0484	0.0746	0.0912
110	0.0282	0.0248	0.0270	0.0494	0.0749	0.0910
120	0.0101	0.0229	0.0264	0.0487	0.0750	0.0909

10 ppm = 256 molar units/ ml enzyme = 0.256 millimolar units/ ml enzyme

20 ppm = 326 molar units/ ml enzyme = 0.326 millimolar units/ ml enzyme

30 ppm = 772 molar units/ ml enzyme = 0.772 millimolar units/ ml enzyme

40 ppm = 1298 molar units/ ml enzyme = 1.298 millimolar units/ ml enzyme

50 ppm = 1616 molar units/ ml enzyme = 1.616 millimolar units/ ml enzyme

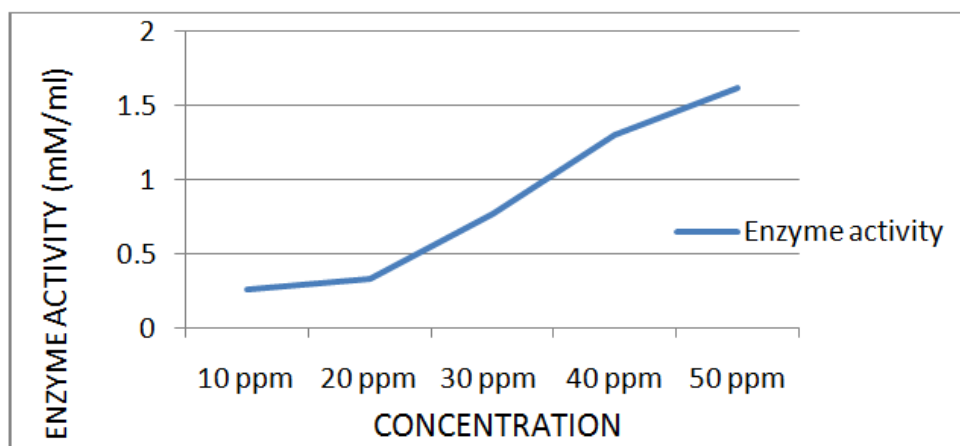


Figure 5: Graph Estimation of Azoreductase by *Chlorella pyrenoidosa*

The azoreductase enzyme activity was observed as concentration was increased, the activity was also increased. The highest enzyme activity was found at 50 ppm of about 1616 molar units/ ml enzyme. The release of azoreductases enzyme was found to be at the faster rate at higher concentrations of dye.

CONCLUSION

The azodye like Malachite green which is toxic to the environment can be reduced by the release of azoreductases enzyme by *Chlorella pyrenoidosa*. As the concentration of dye increased, the release of azoreductases was also higher. At 50 ppm, the azoreductases was found to 1616 molar units/ ml enzyme. This proves the efficiency of *Chlorella pyrenoidosa* in inhibiting the action of azodyes in environment. The other enzymes like SOD and APX were also released by *Chlorella pyrenoidosa* but the release was higher only in 50 and 75 ppm. At higher concentrations of dye, *Chlorella pyrenoidosa* was able to release only azoreductases but not APX and SOD.

ACKNOWLEDGEMENT

The authors record their sincere thanks to Dr. V. Palani, Managing Director of Genewin Biotech, Hosur for providing Technical support and necessary laboratory facilities to carry out this work in their DBT certified laboratory.

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